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Metabolomic Analysis by UAE-GC MS and antioxidant activity of *Salvia hispanica* (L.) seeds grown under different irrigation regimes

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Running Title: Metabolomics and antioxidant activity of chia seeds and the effect of irrigation

Abstract

Chia (*Salvia hispanica* L.) is an emerging crop with a high content of α-linolenic acid and metabolites of industrial and pharmaceutical interest but information on metabolome variations in response to agricultural management is scarce. We investigated the yield and metabolic profile of the seeds of two chia populations, one commercial black (B) and one long-day flowering genotype (G8), in response to two irrigation levels: replacement of 100% ET_0 (I) or rainfed (NI). Seed yield was higher in irrigated plots in G8 only (0.255 kg m^{-2} for I vs 0.184 kg m^{-2} for NI) while it was very low regardless of irrigation in B due to late flowering. Ultrasound assisted extraction (UAE) of seeds followed by gas chromatography-mass spectrometry (GC/MS) analysis showed differences in fatty acids and the major classes of organic compounds due to both genotype and irrigation, especially in the non-polar phase where irrigated samples showed a higher content of α-linolenic and other fatty acids and a lower oleic/linoleic ratio (47.4 in NI vs. 39.6 in I). The antioxidant activity, expressed as trolox equivalent antioxidant capacity (TEAC), ranged from 1.317 ± 0.027 to 2.174 ± 0.010 mmol TEAC/g of defatted chia seed after 2 and 40 min respectively, and was negatively affected by irrigation. The total polyphenolic content (TPC) measured with the Folin-Ciocalteu method, also decreased with irrigation. According to our results irrigation can affect chia yield, metabolome and antioxidant behavior but some of the effects are genotype-dependent.

Keywords: Chia; Polyphenols; TPC; Management; Alpha-linolenic acid; Quality

1. Introduction

Chia (*Salvia hispanica* L.) is an ancient short-day flowering crop with center of origin between Mexico and Guatemala (Cahill, 2004). It was one of the staple crops in pre-Columbian Central America but its diffusion was drastically reduced after the Spanish domination. Chia was re-discovered in the 90s by researchers intending to propose alternative crops to farmers (Coates, 1996; Gentry et al., 1990), and has thereafter spread to several areas of the world, at first due to favorable market placement linked to its nutraceutical properties, and more recently as a source of compounds of cosmetic, medical and industrial interest (Hermoso-Diaz et al., 2014; Lu and Foo, 2002; Muñoz et al., 2013). The *Salvia* genus includes more than 900 species, and in the New World around 500 species belong to the subgenus *Calosphace* (Benth.) and may be grouped in complexes of species with common medicinal activity, mostly related to the content of terpenes in leaves. Jenks and Kim (2013) listed external application (antimicrobic, skin problems and rheumatisms) and internal uses (gastro-intestinal, gynecological and neurological) in the *mirto* complex, primary uses for the respiratory system in the *Nucchu* complex, and primary uses for biliary and kidney problem in the *Li'l ++, Cantueso and Manga-paqui* complexes. They also report that *S. divinorum* Epling and Játiva-M. are the most studied medicinal sage leaves within the *Calosphace* subgenus, due to their use as a hallucinogen by Mexican shamans and their selective kappa-opioid receptor agonist activity. The chia complex, including 18 species besides *S. hispanica* (2013), on the other hand, is the most renowned for the production of indehiscent dry fruits, commonly referred to as "seeds", rich in oil.

Chia seeds are one of the richest natural sources of omega 3 fatty acids (Ayerza and Coates, 2011; Ayerza, 1995) and show a high content of protein with a balanced composition in essential amino acids (Ayerza, 2013) and fiber (Capitani et al., 2012). A part of the fiber is located in the outer cells of the fruit and is extruded at the fruit surface upon hydration (Muñoz et al., 2012), forming a mucilaginous capsule with rheological properties that make it promising for industrial and medical uses: it is highly hygroscopic, viscous and adhesive (Švec et al., 2016). Many antioxidants have been identified in chia seeds, extracted oil and mucilage, especially phenolic acids and flavonoids, besides poly-unsaturated fatty acids (Amato et al., 2015; da Silva Marineli et al., 2014). Chia seeds or their products are therefore increasingly proposed not only as food but also as a component for biodegradable film (Capitani et al., 2016; Muñoz et al., 2012), thickening agents (Coelho and de las Mercedes Salas-Mellado, Myriam, 2015; Felisberto et al., 2015; Iglesias-Puig and Haros, 2013; Menga et al., 2017), anti-corrosive agents (Hermoso-Diaz et al., 2014), cosmetics (Muñoz et al., 2013) and medicaments (Vuksan et al., 2010). A strong variation in chia seeds' composition has been reported: for instance, oil content ranges from little over 20% to over 36% (Ayerza and Coates, 2004;

Ayerza, 1995; Coelho and de las Mercedes Salas-Mellado, Myriam, 2014; da Silva Marineli et al., 2014; Ixtaina et al., 2011). Variability has mainly been researched in relation to genotype and environment: Ayerza (2009) reports a range in total fat content from 25.93% to 33.50% for the same genotype of chia grown in five different environments. The fatty acids profile, especially the content of α-linolenic acid, is also affected by elevation in seeds of this species, even within the same genotype (Ayerza and Coates, 2011; Martínez-Cruz and Paredes-López, 2014). This is probably largely due to thermal effects of elevation, as reported by Ayerza and Coates (2004) who found a correlation between temperature and chia oil fatty acids measured across different environments; they concluded that levels of fatty acids' unsaturation in chia increase at cooler temperatures, as observed for other oil seed crops due to saturase-desaturase dynamics. In an experiment conducted across different countries in America, Ayerza (2009) reports a direct relationship between elevation and oil content and an inverse relationship between elevation and the content of proteins. Several environmental variables might be involved besides temperature, including soil properties and a negative correlation between oil and protein as found in other crops. Ayerza and Coates (2011) suggest that the relationships of oil and protein content and oil saturation with elevation are strong enough that they could be used to trace the growing environment of chia. Ayerza (2009) also found differences in protein content for the same genotypes grown in different environments, but could not prove differences among genotypes within a site, except for one variety at one site. In a further study, Ayerza (2013) could not find significant differences between two genotypes of different seed coat color for protein, oil, fiber, amino acids, and antioxidant content. (Silva et al., 2016) found that a white and a black seed crop with the same seed yield produced different amounts of unsaturated fatty acids: the white seed genotype yielded more linoleic and α-linolenic acids (6.0 and 17.0 kg ha^{-1} respectively) than the black seed one (4.4 and 16.7 kg ha^{-1} respectively). De Falco et al. (2017b) studied the metabolic profile of the seeds of seven chia populations, including commercial and early flowering mutant genotypes, and showed significant differences in the metabolic fingerprinting of the different populations using nuclear magnetic resonance (NMR) and chemometrics. An investigation of the metabolome with gas chromatography-mass spectrometry has not yet been performed on chia seeds after agronomic management.

Very little information is available about the variation in chia seeds composition with agronomic management. Amato et al. (2015) compared nitrogen fertilization regimes on chia seed composition and found a higher *p*-anisidine value, content of phenols and oxidative stability in plots fertilized with organic nitrogen only, whereas the addition of mineral nitrogen in topdressing increased free acidity, chlorophyll and carotenoids content. De Falco et al. (2017b) reported that the effect of mineral nitrogen supply on chia positively affects the content of aliphatic free amino acids, and negatively

that of the main carbohydrates and flavonoids. Heuer et al. (2002) found that salinity of irrigation water decreases the oil content of chia seeds and increased their content of palmitic and linoleic acids. Irrigation is one of the major agronomical factors conditioning crop yield and composition, and namely that of oilseeds (Flagella et al., 2002). Silva et al. (2016) did not find a significant effect of irrigation on chia seed yield and content of linoleic and α-linolenic acids. However authors point out that their experiment was conducted using short-day flowering genotypes at a latitude higher than optimal; due to photoperiod sensitivity flowering was delayed and temperatures during seed maturation were too low to allow complete grain filling. In this condition, temperature and not water was the limiting factor and even fully irrigated plots yielded poorly. More information is therefore needed on the response of chia to irrigation taking photoperiod sensitivity into account. The objective of this research was to study the response of chia to irrigation with the hypothesis that irrigation affects the yield of chia seeds, the fatty acid profile and the production of secondary metabolites found in the polar and non-polar extracts. The hypothesis that responses to irrigation are different in short-day and long-day flowering genotypes at high latitudes was also tested by using a short-day commercial chia seed source and a long-day flowering mutant.

2. Materials and methods

2.1 Plant material

Black chia (*Salvia hispanica* L.) seeds (B) were obtained from a commercial retailer (Eichenhain-Hofgeismar-DE) and seeds of one long-day flowering mutant genotype (G8) were obtained as described in Jamboonsri et al. (2012) and were kindly supplied through an agreement between the University of Basilicata and the University of Kentucky (US).

2.2 Growth conditions

Plants were grown in Basilicata (Southern Italy-Lat. N 40° 51′ 37,59″ Lon. E 15° 38′ 49,43″) on a Luvi-vertic Phaeozem (IUSS, 2007), loam soil (43.6% of sand, 34.2% silt and 22.1% clay) in the period June-December 2014. Soil water content was 0.279 g g^{-1} at -0.03 MPa and 0.137 g g^{-1} at -1.5 MPa. A field factorial randomized block design with three replications was established to test the two genotypes B and G8 with two levels of irrigation:

NI = no irrigation

I = nonlimiting water supply. We provided 100% of ET_0 corresponding to the evaporative demand of the atmosphere (Allen et al., 1998) measured with a TE-ETG atmometer (Tecnoel, Rm Italy).

The irrigation system was drip with pre-installed emitter lines with drippers at 200 mm distance and maximum flow rate 6.6x10-^{5} m^{3} s^{-1} at 1x10^{5} Pa. Plot size was 8 x 5 m with a density of 20 plants m^{-2}. The crop was sown on June 26, 2014. Precipitation and irrigation amounts are shown in Fig.1; total precipitation was 197 mm during the experiment and all plots received the same initial regime with 52 mm of irrigation to help crop establishment. Treatments were differentiated at 51 DAS (day after sowing) when the I treatment was left in rainfed conditions and the I treatment was irrigated receiving 172 mm of further irrigation. Evaporation was read daily and irrigation was performed when a set amount was reached. In order to cover the crop's needs for root establishment and full deployment of deep rooting potential, irrigation schedule and amount ranged from irrigation with around 4.5 mm every 4 days up to 15 DAS, to an irrigation amount of around 14 mm on average per irrigation thereafter. This corresponded to a number and timing of irrigations varying according to evaporation and the timing of precipitation: 5 irrigations in July (with a maximum of 10 days without irrigation due to precipitation), 10 in August (every 2-4 days), 2 in September (with a maximum of 26 days without irrigation due to precipitation) and October (with a maximum of 21 days without irrigation due to precipitation). Seeds of G8 and B were harvested at 132 and 173 DAS respectively, on one sample of 20 plants per replication. A trench dug after harvest showed roots up to 2 m and a total soil depth exceeding 3 m.

2.3 Chemicals and reagents

The reagents used for the extraction procedure and chemical characterization, namely anhydrous methanol (99.8%), anhydrous *n*-hexane (95%), 2,2`-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), derivatizating agents methoxyamine hydrochloride and N-methyl-N-trimethylsilyltrifuoroacetamide (MSTFA) were obtained from Sigma-Aldrich (Dorset, UK). *N, O*-Bis(trimethylsilyl)trifluoroacetamide, Trimethylchlorosilane (BSTFA with 1% TMCS) was purchased from Supelco Analytical (Bellefonte, PA). Pyridine, anhydrous sodium carbonate, Folin-Ciocalteu's reagent and gallic acid were obtained from Fischer Scientific (Loughborough, UK). Sugars, amino acids, organic acids and polyphenols used for identification and quantification were purchased from Sigma-Aldrich (Dorset, UK).

2.4 Extraction procedure of the organic phase

Chia seeds were strained in order to remove extraneous matter such as dust and straw. The clean seeds were blended in a laboratory mill (IKA Works MF10, Scotland, UK) in order to obtain a fine powder of the organic material. Subsequently, the powder (15g) was extracted with 80 ml of *n*-hexane for 2 h under stirring. The mixture was centrifuged at 3700rpm for 10 min and the supernatant was

immediately stored at -80°C in the dark for later analysis. The pellet was washed twice with 20ml of *n*-hexane and then centrifuged at 3700rpm for 10 min. The supernatant was added to the previous fraction and the leftover pellet was left overnight in a fume hood in order to remove the excess solvent.

2.5 Ultrasound assisted extraction (UAE)

The defatted chia seeds (10g) were extracted with 100ml of methanol/water (60:40). Sonication was performed at 20 kHz with 50% power using a Fischer Scientific Ultrasound (model FB705, 700W, 2000 Park Lane, Pittsburgh, PA) with continuous stirring. The probe was a horn-type (model CL-334), which was kept at constant depth in the mixture using a 250ml glass beaker of standard dimensions. During the extraction, the temperature was monitored and kept constant (25°C ± 1) using a thermostatic bath. Samples (5ml) were collected after 2, 20, 40 min and centrifuged at 2500rpm for 10 min. The supernatant was stored at -80ºC for later analysis.

2.6 Total polyphenol content

Total polyphenolic content (TPC) was determined by spectrophotometry according to the method described by Singleton and Rossi (1965) with some modifications: 125µl of diluted sample (1:10) were mixed with 500µl of distilled water and 125µl of Folin-Ciocalteu reagent. After 6 min, 1.25ml of a 7.5% sodium carbonate solution were added to the mixture and brought to a final volume of 3ml with distilled water. The test tubes were then allowed to stand in the dark for 90 min at room temperature. The absorbance was read at 760nm (Thermo Scientific Genesys 10S UV-Vis Spectrophotometer) and TPC was expressed in terms of gallic acid equivalents (GAE/g). A calibration curve ranging from 20 to 200µg ml^{-1} was used to quantify the TPC content in the seed extracts. All determinations were performed in triplicate.

2.7 Antioxidant activity

The free radical-scavenging activity was determined according to Re et al., (1999) using the reduction of radical cation 2,2`-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$). A mixture of 2.5ml of 7 mM ABTS and 44µl of 140mM potassium persulfate was prepared and left overnight in the dark. The spectrophotometer wavelength was set at 734nm. The stock solution of ABTS was diluted to 1:80 until a final OD (optical density) reached a value between 0.7 and 0.8nm. Samples were diluted 1:10 and 100µl were added to 1ml of ABTS solution. After 2.5 min the reduction was measured as the percentage of inhibition. Results were expressed in mmol Trolox

equivalent antioxidant capacity (TEAC/g) and referred to a calibration curve ranging from 25 to 250µM. All determinations were performed in triplicate.

2.8 Derivatization and GC/MS analysis

In order to obtain volatile and stable compounds, both polar and non-polar extracts were derivatized before analysis by GC/MS. For this purpose, the organic phase was dried under nitrogen stream and a stock solution (500ppm) was prepared. A subsample of 75µl was dissolved in 300µl of pyridine/BSTFA + 1% TMCS (1:1). The vials were vortexed, left at 25°C for 15 min and analyzed by GC/MS (Shareef et al., 2006). The metabolomic analysis of the polar extract needed a two-step process, starting with oximation to reduce tautomerism of aldehydes and ketones, followed by OH, SH and NH silylation (Gullberg et al., 2004). An aliquot (200µl) of diluted sample (1:50) was evaporated to dryness in a vacuum centrifuge (Eppendorf Concentrator 5301) and oxymated with 50µl of methoxyamine hydrochloride (20mg ml^{-1}) in pyridine at 60°C for 45 min. Samples were then silylated with MSTFA at 60°C for 45 min. Both polar and non-polar extracts were analyzed in a similar way by gas chromatography - mass spectrophotometry. Derivatized samples were injected (1µl) in a pulsed splitless mode into an Agilent-7820A GC system with 5977E MSD operating in EI mode at 70eV. The system was equipped with a 30m x 0.25mm id fused-silica capillary column with 0.25µm HP-5MS stationary phase (Agilent technologies, UK). The injection temperature was set at 270°C. Helium was used as carrier gas at a constant flow rate of 1ml min^{-1}. Separation of the non-polar extract was achieved using a temperature program of 80°C for 1 min, then ramped at 10°C/min to 320°C and held for 1 min. The analysis of the polar compounds was performed under the following temperature program: 2 min of isothermal heating at 70°C, followed by a 10°C/min oven temperature ramp to 320°C, and a final 2 min at 320°C. The system was then temperature equilibrated for 1 min at 70°C before injection of the next sample. All spectra were recorded in the mass range 50 to 800 *m/z*. Both chromatograms and mass spectra were evaluated using the MassHunter Qualitative Analysis B.07.00 (Agilent Technologies, CA, USA). Mass spectra of all detected compounds were compared with standard compounds and with spectra in National Institute of Standard and Technologies library NIST MS Search 2.2 (https://www.nist.gov). Data was processed with the AMDIS (Agilent Technologies, CA, USA) software to deconvolute co-eluting peaks. Artifact peaks, such as peaks due to derivatizing agents, were not considered in the final analyses. Peak areas of multiple peaks belonging to the same compound were summed together. The relative amount of separated metabolites was calculated from Total Ion Chromatography (TIC) by the computerized integrator and with the internal standard, malonic acid and 1-oleoyl-rac-glycerol, added to polar and non-polar extracts respectively.

2.9 Statistical analysis

Relative quantification was done by integrating the peak areas of the chromatographic profiles for each compound and normalizing the data to the internal standards. The effect of genotype, irrigation and their interaction on the chemical composition of chia seeds was evaluated through the analysis of variance with the R software (RDevelopment, 2012) after checking for normality and homoscedasticity of data.

3. Results and Discussion

3.1 Seed production and chemical properties

The genotype x irrigation interaction for seed yield is reported in Fig. 1. Yields of G8 were in the high yielding end of the range of productive data reported for chia worldwide (Bochicchio et al., 2015a) and were significantly higher than those of B. Irrigated plots yielded more than NI but statistical significance of this difference was reached in G8 only. Results confirm first reports of low yields from traditional genotypes of chia grown at high latitudes (Bochicchio et al., 2015b; Silva et al., 2016). Traditional genotypes are microthermal and short-day flowering, therefore are sown in late spring and cannot flower until late summer; seed ripening occurs in fall-winter and is hampered by low temperatures. Besides low yields, this explains the lack of significant effects of irrigation on the short-day flowering genotype since temperature during ripening becomes the limiting factor for this chia type and makes it difficult to detect yield differences due to other factors. This confirms the results of Silva et al. (2016) who also report non-significant irrigation effects on a short-day flowering genotype grown at high latitude.

Oil yield (Fig. 1) was 39.6% on average without significant differences between treatments. This value is in the high range of chia seeds oil content (Bochicchio et al., 2015a).

The TPC results, expressed as mg GAE/g of defatted chia seed, were in agreement with Amato et al. (2015) and with other previous reports (Coelho and de las Mercedes Salas-Mellado, Myriam, 2014; Reyes-Caudillo et al., 2008) but lower compared to data reported by da Silva Marineli et al. (2014). The polyphenol content increased after 40 min of UAE ($p<0.01$). Although in many cases values were lower in irrigated treatments, differences were statistically significant ($p<0.01$) only for the commercial variety at 2 minutes, and sample variability did not allow significance to be reached in the other instances. The general trend of our data agrees with literature reports of a negative effect of irrigation on TPC for different species (Dag et al., 2008; Esteban et al., 2001).

3.2 Antioxidant activity

The antioxidant activity of chia seeds was also evaluated and Fig. 1 shows the TEAC results for Black chia and G8 seeds. The values ranged from 1.317 ± 0.027 to 2.174 ± 0.010 mmol TEAC/g of defatted chia seed, measured after 2 and 40 min, respectively. These results are in agreement with Sargi et al. (2013), but higher than those reported by other authors (Capitani et al., 2012; Vázquez-Ovando et al., 2009). Values were lower in irrigated treatments, but differences were statistically significant ($p<0.01$) only for G8 at 2 minutes. In many plant species, the amount of antioxidants and/or antioxidant activity is shown to increase (Wu et al., 2017) or remain unaffected (Kyraleou et al., 2016) with reduced water supply. Zhang and Kirkham (1996) show that the degree to which the activities of antioxidant enzymes and the amount of antioxidants change under drought stress is variable with plant species. No reports of chia antioxidant activity in response to irrigation are available, but Amato et al. (2015) found a reduction in oxidative stability and phenols in response to mineral nitrogen fertilization.

3.3 Metabolite profile

A whole metabolome profile of two different genotypes (G8 and Black chia) of *Salvia hispanica* L. seeds was evaluated by GC/MS analysis. All compounds from the polar and non-polar extracts with their respective retention times and *m/z* values are listed in Table 1, and representative total ion chromatograms (TIC) for both fractions are reported in Fig. 2. Our assignments from GC/MS are in agreement with those obtained using NMR and chemometrics by de Falco et al. (2017b) who performed a metabolomic fingerprinting of different populations of *S. hispanica* including G8 and Black chia seeds. The GC/MS approach, however, allowed us to identify compounds which were not detected by previous work such as the 10-heptadecenoic acid (17:1) and the comparison of irrigation treatments allowed us to detect responses (Table 2) which were not investigated by other experiments.

3.4 Non-polar phase

The main fatty acids detected in chia seeds were linolenic acid (18:3), linoleic acid (18:2), stearic acid (18:0), palmitic acid (16:0) and oleic acid (18:1), determined as trimethylsilyl (TMS) derivatives. These compounds were previously detected in other reports (Amato et al., 2015; Peiretti and Gai, 2009). A preliminary analysis of chromatograms showed C18:3 as the most abundant fatty acid in all samples. On the contrary, 10-heptadecenoic acid (17:1) was detected in the lowest amount. This

298 compound was also reported as one of the least abundant by Segura-Campos et al. (2014) and da
299 Silva Marineli et al. (2014).
300 MS spectrum of saturated fatty acids trimethylsilyl esters such as C16:0 and C18:0 have a base peak
301 at *m/z* 313 and 341 respectively, which represents the loss of methyl group from TMS ester group,
302 while *m/z* 132 represents the McLafferty rearrangement ion. In the MS spectrum of monounsaturated
303 and polyunsaturated TMS fatty acids, such as C18:1, C18:2 and C18:3 characteristic peaks for each
304 of them were detected at *m/z* 339, 337 and 335, respectively. Also in these chromatograms, a base
305 peak at *m/z* 73, due to TMS, was always detected. As shown in Fig. 3, the most abundant fatty acid
306 is α-linolenic acid (C18:3) in all samples, and this is in agreement with those of other reports (Coelho
307 and de las Mercedes Salas-Mellado, Myriam, 2014; de Falco et al., 2017a). Analysis of variance on
308 the non-polar fraction (Table 2) shows that the main effects of genotype and irrigation were
309 significant in many cases. In particular, the total amount of fatty acids was found higher in G8 than
310 in B, except for C17:1 which was higher in B, and oleic acid (C18:1) and glycerol monostearate
311 (GMS) which were not significantly different between genotypes. Although irrigation treatment
312 affected the fatty acids composition of both genotypes of chia seeds, an interaction was significant in
313 C18:1 where values of the irrigated treatment were higher than NI only for G8, and in GMS where
314 I<NI in G8 and I>NI in B. Silva et al. (2016) reported that irrigation did not affect significantly the
315 content of linoleic and α-linolenic acids of chia, but their experiment tested less extreme levels of
316 irrigation, ranging between 40% and 100% of ET_0, whereas in our case 100% of ET_0 is compared
317 with rainfed conditions. No metabolomic study on the effect of irrigation on chia seeds is found in
318 the literature, and few reports on the effect on single metabolites are available: Silva et al. (2016) did
319 not find differences in linoleic and α-linolenic acids content of chia seeds grown in different irrigation
320 regimes, and Heuer et al. (2002) found an increase in the levels of palmitic and linoleic acids in
321 response to salinity in irrigation water. In other species irrigation is reported to affect fatty acids
322 composition but results are often contradictory. Erdemoglu et al. (2003) found a decrease in the
323 content of linoleic and oleic acids with irrigation in sunflower seed oil. Sezen et al. (2011) found an
324 increase in linoleic, palmitic and stearic acid concentrations with irrigation. Bellaloui et al. (2015)
325 reported that irrigation affects soybean oil composition differently according to the degree and stage
326 of differentiation of water treatments. Ayerza (2009) reports a negative correlation of the percentages
327 of oleic and linoleic acid in chia samples from different environments. This is found in many crops,
328 due to the dynamics of oleate desaturases: an increase of desaturation in cold conditions causes a
329 decrease of the oleic/linoleic acids ratio in cooler environmental temperatures (Aparicio et al., 1994).
330 In our data, although the amount of many fatty acids increases in response to irrigation, the response
331 is proportionally lower in oleic acid, and therefore the oleic/linoleic ratio decreases from 47.4 in the

rainfed samples to 39.6 in the irrigated treatments. Flagella et al. (2002) observed a decrease in the oleic/linoleic acid ratio in sunflower in response to irrigation, and suggested that a possible thermal effect of irrigation may have affected the activity of oleate desaturase.

3.5 Polar phase

In the aqueous extracts sugars are the principal class of compounds and the disaccharide sucrose (*m/z* 361) represents the major component followed by methyl galactose as the major monosaccharide component (Table 1). Other sugars identified were glucose, galactose, fructose, mannitol and gluconic acids. In particular, TMS derivatives of monosaccharides such as glucose and galactose showed a very similar GC/MS profiles, due to their stereoisomery, with characteristic ions observed at *m/z* 147, 205, 319 and 364. The final identification of these compounds was achieved by comparing their elution order with literature data (Gómez-González et al., 2010) and by injection of standard samples. Other compounds detected were the polyphenol caffeic acid, the polyol myo-inositol and a series of carboxylic acids and amino acids (Table 1). A base peak at *m/z* 73, typical of silylated compounds, was always detected in the chromatograms due to the [$(CH_3)_3Si$] group. In the polar fraction (Fig. 4) sucrose (Sucr) and methyl-galactoside (mGal) are the most abundant sugars present in all samples, while within organic acids, lactic acid (LA) and citric acid (CI) have the highest value, followed by quinic acid (QUI).

Caffeic acid (CA) did not exhibit variation within genotype, but even if statistically not significant, its level is higher in Black chia than G8. Values were more variable than those of the non-polar phase, and statistical significance of the differences between genotype and irrigation treatments was not reached for many compounds (Fig. 4). A genotype effect was significant for some compounds (Fig. 4): G8 showed a significantly higher amount of lactic acid (LA), benzoic acid (BE) and serine (Ser), while B showed a significantly higher amount of gluconic acid (GLUC), arabitol (Ara) and sugars, in particular mannose (Man). The overall effects of irrigation were not significant but interactions were found for some compounds (Fig. 4): irrigated samples showed significantly higher values of glycine (Gly) in G8 only, and significantly lower values of Asp and phenylalanine (Phe) in B only. No reports of chia polar extracts variation in response to irrigation are available in the literature. The only other data regarding agricultural management effects on chia seeds whole metabolome are related to nitrogen fertilization and point out a positive effect of mineral nitrogen topdressing on the content of aliphatic free amino acids, a reduction of carbohydrates and flavonoids and no effect on the pools of caffeoyl derivatives and organic acids (de Falco et al., 2017b).

4. Conclusion

This work provides a high-throughput analysis of metabolomic fingerprinting including total polyphenolic content (TPC) and antioxidant activity (TEAC), on commercial black chia and early flowering G8 seeds. The analytical approach performed by UAE-GC MS allowed detection and quantification of a high number of metabolites.

The aim of the paper was to evaluate the difference in organic compounds between a commercial genotype, black chia, and the recently developed mutant G8. The species were grown at different levels of irrigation to evaluate the effect of water supply on the metabolite content. Results showed an increase of TPC and antioxidant activity (expressed as TEAC) in all samples relative to irrigation treatment or variety after 40 minutes of UAE. On the contrary a decrease of TPC and TEAC levels was observed after irrigation treatments. With regards to the non-polar phase, quantitative analysis showed a higher yield and content of many fatty acids including ω-3 (α-linolenic), in the early flowering G8 mutant, with a decrease of the ratio of oleic/linoleic acids. Concerning the polar fraction, sugars were found as the main metabolites with sucrose and methyl galactose as the major components. The genotype effect has more influence than the irrigation treatment on the aqueous extract. G8 showed significantly higher amounts of some organic acids and amino acids, such us LA, BE and Ser while GLUC, Ara and Mann are present in lower amounts.

Gas chromatography-mass spectrometry can provide a detailed metabolic profile of chia seeds extracted with ultrasound. Furthermore, this study highlighted for the first time the effects of irrigation on the metabolome of a late flowering and an early flowering mutant chia genotypes.

In addition, this approach proved that chia seeds of mutant genotypes can be cultivated at high latitude without loss in nutraceuticals. Within mutants, G8 can be proposed as excellent early-flowering genotype.

The obtained data indicate that metabolomics could be used as monitoring technique to control the agronomic management and its non-invasive features making it an ideal tool for crop production.

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Notes

The authors declare no competing financial interest.

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566 TABLES

567 **Table 1** Polar and non-polar metabolites assigned in Chia seeds by GC-MS

Peak	Detected metabolite	Abbreviation	RT (min)	Molecular formula	*m/z*
Polar					
1	Lactic Acid	LA	7.25	$C_9H_{22}O_3Si_2$	219, 191, 147, 133, 117, 73, 45
2	Methyl 2-ethyl malonate	Me-MA	7.32	$C_9H_{18}O_4Si$	175, 89, 73
3	L-Alanine, N-methyl-N-(trifluoroacetyl)-, butyl ester	Ala, Nm-Tfa-Obu	9.99	$C_{10}H_{16}F_3NO_3$	154, 110
4	Benzoic Acid	BE	10.03	$C_{10}H_{14}O_2Si$	194, 179, 135, 105, 77
5	Glycerol	GLY	10.46	$C_{12}H_{32}O_3Si_3$	218, 205, 147, 117, 89, 73, 45
6	L-serine	Ser	11.74	$C_{12}H_{31}NO_3Si_3$	218, 204, 147, 100, 73
7	L-Threonine	Thr	12.14	$C_{13}H_{33}NO_3Si_3$	291, 218, 147, 117, 73
8	N-α-Acetyl-L-Lysine	AcLys	13.04	$C_{17}H_{40}N_2O_3Si_3$	404, 287, 73
9	Malic acid	MA	13.52	$C_{13}H_{30}O_5Si_3$	245, 233, 147, 133, 73
10	L-Aspartic acid	Asp	13.97	$C_{13}H_{31}NO_4Si_3$	232, 218, 147, 100, 73
11	L-5-Oxoproline	PCA	14.00	$C_{11}H_{23}NO_3Si_2$	258, 230, 156, 133, 73, 45
12	L-Glutamic acid	Glu	15.21	$C_{14}H_{33}NO_4Si_3$	246, 147, 128, 73
13	Phenylalanine	Phe	15.35	$C_{15}H_{27}NO_2Si_2$	218, 192, 147, 100, 73
14	Tartaric acid	TA	15.54	$C_{28}H_{62}O_6Si_4$	549, 417, 389, 147, 73
15	Citric acid	CI	17.69	$C_{18}H_{40}O_7Si_4$	273, 147, 73, 45
16	Methyl galactoside	mGal	17.77	$C_{19}H_{46}O_6Si_4$	243, 217, 204, 133, 73
17	Quininic acid	QUI	18.24	$C_{22}H_{52}O_6Si_5$	345, 255, 191, 147, 73
18	D-Fructose MEOX	FRU	18.37	$C_{22}H_{55}NO_6Si_5$	217, 307
19	Arabitol	Ara	18.48	$C_{20}H_{52}O_5Si_5$	307, 277, 217, 189, 147, 103, 73

20	D-Galactose MEOX	Gal	18.61	$C_{22}H_{55}NO_6Si_5$	319, 205, 147, 103, 73
21	D-Glucose MEOX	Glc	18.67; 18.86	$C_{22}H_{55}NO_6Si_5$	364, 319, 205, 147, 73
22	D-Mannitol	MAN	19.04	$C_{24}H_{62}O_6Si_6$	421, 345, 319, 205, 147, 103, 73
23	D-Gluconic acid	GLUC	19.77	$C_{24}H_{60}O_7Si_6$	333, 292, 205, 147, 103, 73
24	Trimethylsilyl catechollactate tris(trimethylsilyl) ether	Cat	20.30	$C_{21}H_{42}O_5Si_4$	396, 267, 179, 147, 73
25	Myo-Inositol	Myo	20.69	$C_{24}H_{60}O_6Si_6$	305, 217, 147, 129, 73
26	Caffeic acid	CA	20.91	$C_{18}H_{32}O_4Si_3$	396, 381, 219, 191, 73
27	Sucrose	Sucr	25.66	$C_{36}H_{86}O_{11}Si_8$	437, 361, 319, 271, 217, 147, 103, 73
Apolar					
28	Palmitic Acid	C16:0	17.88	$C_{19}H_{40}O_2Si$	328, 313, 145, 117, 73
29	Linoleic acid	C18:2	19.54	$C_{21}H_{40}O_2Si$	337, 129, 95, 75, 73
30	α-Linolenic acid	C18:3	19.61	$C_{21}H_{38}O_2Si$	335, 129, 95, 75, 73
31	Stearic acid	C18:0	19.80	$C_{21}H_{44}O_2Si$	356, 341, 132, 117, 73
32	Oleic acid	C18:1	22.07	$C_{21}H_{42}O_2Si$	354, 339, 129, 117, 73
33	10-Heptadecenoic acid	C17:1	24.05	$C_{20}H_{40}O_2Si$	340, 325, 145, 129, 117, 73
34	Glycerol monostearate	GMS	24.34	$C_{27}H_{58}O_4Si_2$	487, 399, 147, 73

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570 **Table 2** Analysis of variance on polar and non-polar fraction to evaluate the effect of genotype and
571 irrigation on Chia seeds

Compounds	Genotype	Irrigation	Genotype x Irrigation
Polar			
LA	p<0.05	n.s.	p<0.05
Me-MA	n.s.	n.s.	n.s.
Ala-Obu	n.s.	n.s.	n.s.
BE	p<0.05	n.s.	n.s.
GLY	n.s.	n.s.	p<0.05
Ser	p<0.05	n.s.	n.s.
Thr	n.s.	n.s.	n.s.
AcLys	n.s.	n.s.	n.s.
MA	n.s.	n.s.	n.s.
Asp	p<0.01	n.s.	p<0.05
PCA	n.s.	n.s.	n.s.
Glu	n.s.	n.s.	n.s.
Phe	n.s.	n.s.	p<0.05
TA	n.s.	n.s.	n.s.
CI	n.s.	n.s.	n.s.
mGal	n.s.	n.s.	n.s.
QUI	n.s.	n.s.	n.s.
FRU	n.s.	n.s.	n.s.
Ara	p<0.05	n.s.	n.s.
Gal	n.s.	n.s.	n.s.
Glc	n.s.	n.s.	n.s.
MAN	p<0.01	n.s.	n.s.
GLUC	p<0.01	n.s.	n.s.
Cat	n.s.	n.s.	n.s.
Myo	n.s.	n.s.	n.s.
CA	n.s.	n.s.	n.s.
Sucr	n.s.	n.s.	n.s.
GABA	n.s.	n.s.	n.s.
Non-polar			
C16:0	P<0.05	P<0.001	n.s.
C18:2	P<0.01	P<0.0001	n.s.
C18:3	P<0.01	P<0.0001	n.s.
C18:0	P<0.01	P<0.0001	n.s.
C18:1	n.s.	P<0.0001	P<0.01
C17:1	P<0.01	P<0.0001	n.s.
GMS	n.s.	n.s.	P<0.01

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FIGURE LEGENDS

Figure 1. Growth environment data and interaction of genotype x irrigation for yield and chemical properties of Chia seeds (*Salvia hispanica* L.). Left: temperature, precipitation and irrigation amounts during crop growth (top); seed yield and oil content (bottom). Different lower case letters indicate significant differences ($p<0.05$) at the post-hoc Tukey's test. Right: Total polyphenol content (top) and Antioxidant activity (bottom) of defatted Chia seeds. Within each time different upper case letters indicate highly significant differences ($p<0.01$) at the post-hoc Tukey's test.

Figure 2. Representative TIC of the polar (A) and non-polar (B) fractions of Chia seeds extracts. Peaks correspond to numbering of compounds in Table 1

Figure 3. Metabolites belonging to different classes of compounds of non-polar extract of Chia seeds detected by GC/MS analysis. The Y-axis indicates the relative quantification obtained by integrating the peak areas of the chromatographic profiles for each compound and normalizing the data to the internal standards. Top left: main effects of genotype; top right: main effect of irrigation; bottom left: interaction of genotype x irrigation. Within each compound different upper case letters indicate highly significant differences ($p<0.01$) and different lower case letters indicate significant differences ($p<0.05$) at the analysis of variance for main effects and at the post-hoc Tukey's test for the interaction.

Figure 4. Metabolites belonging to different classes of compounds of non-polar extract of Chia seeds detected by GC/MS analysis. The Y-axis indicates the relative quantification obtained by integrating the peak areas of the chromatographic profiles for each compound and normalizing the data to the internal standards. Top left: overall average for compounds not significantly different between treatments; top right: main effect of genotype for compounds significantly different between G8 and B; bottom left: interaction of genotype x irrigation; within each compound different lower case letters indicate significant differences ($p<0.05$) at the post-hoc Tukey's test for the interaction.

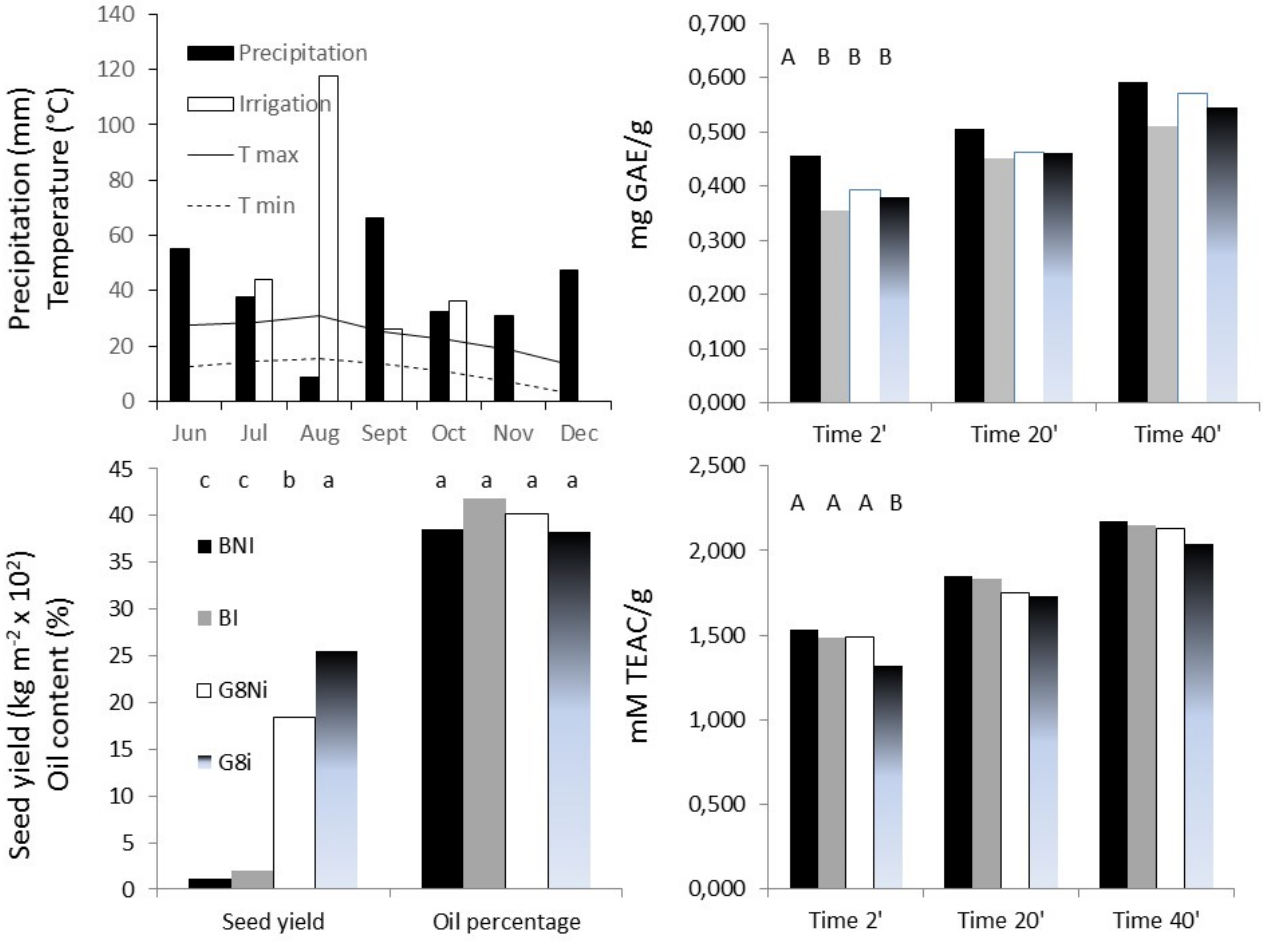
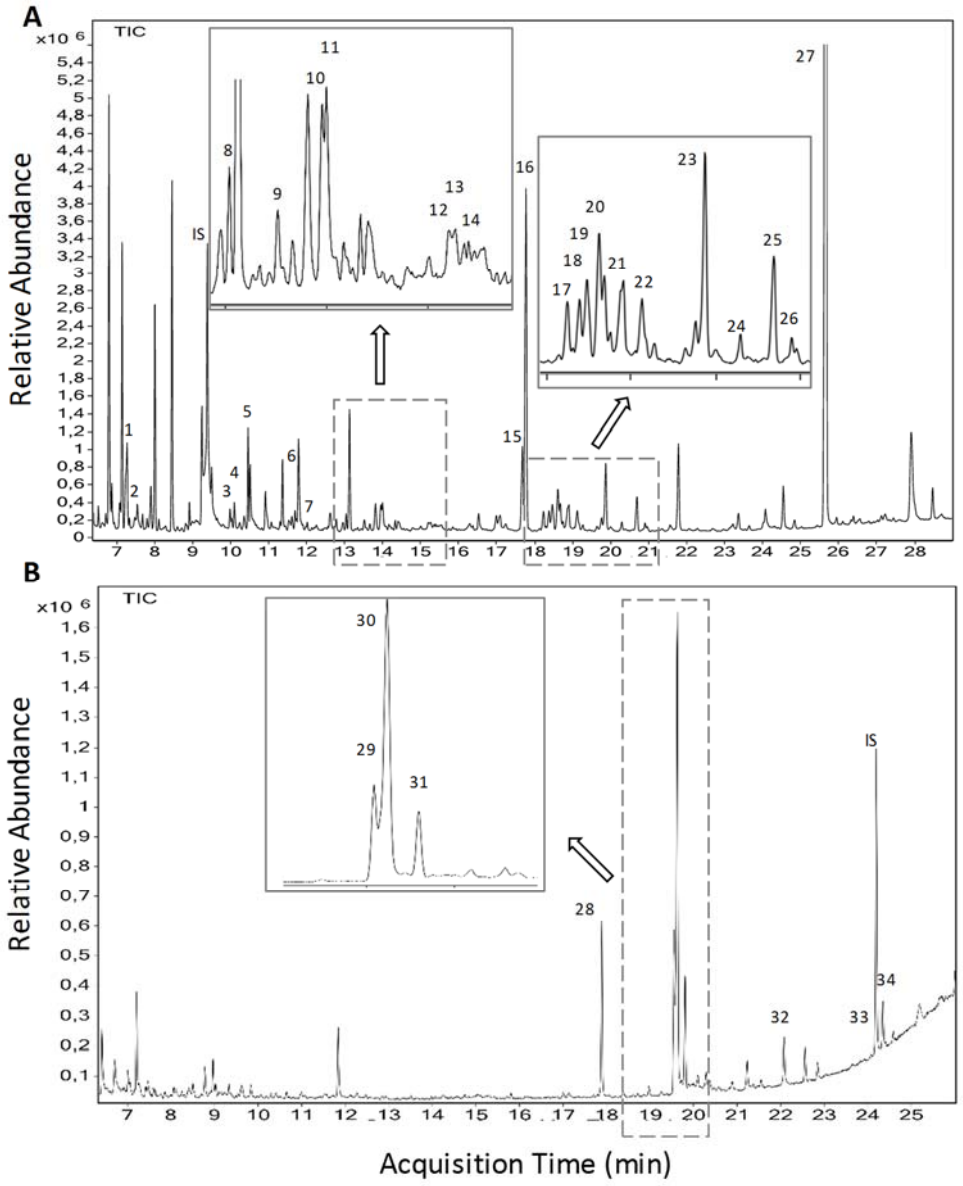

Precipitation (mm)
Temperature (°C)
Precipitation
Irrigation
T max
T min
Jun
Jul
Aug
Sept
Oct
Nov
Dec
mg GAE/g
A B B B
Time 2'
Time 20'
Time 40'
Seed yield (kg m-2 x 10²)
Oil content (%)
c c b a
a a a a
BNI
BI
G8Ni
G8i
Seed yield
Oil percentage
mM TEAC/g
A A A B
Time 2'
Time 20'
Time 40'

Figure 1


A
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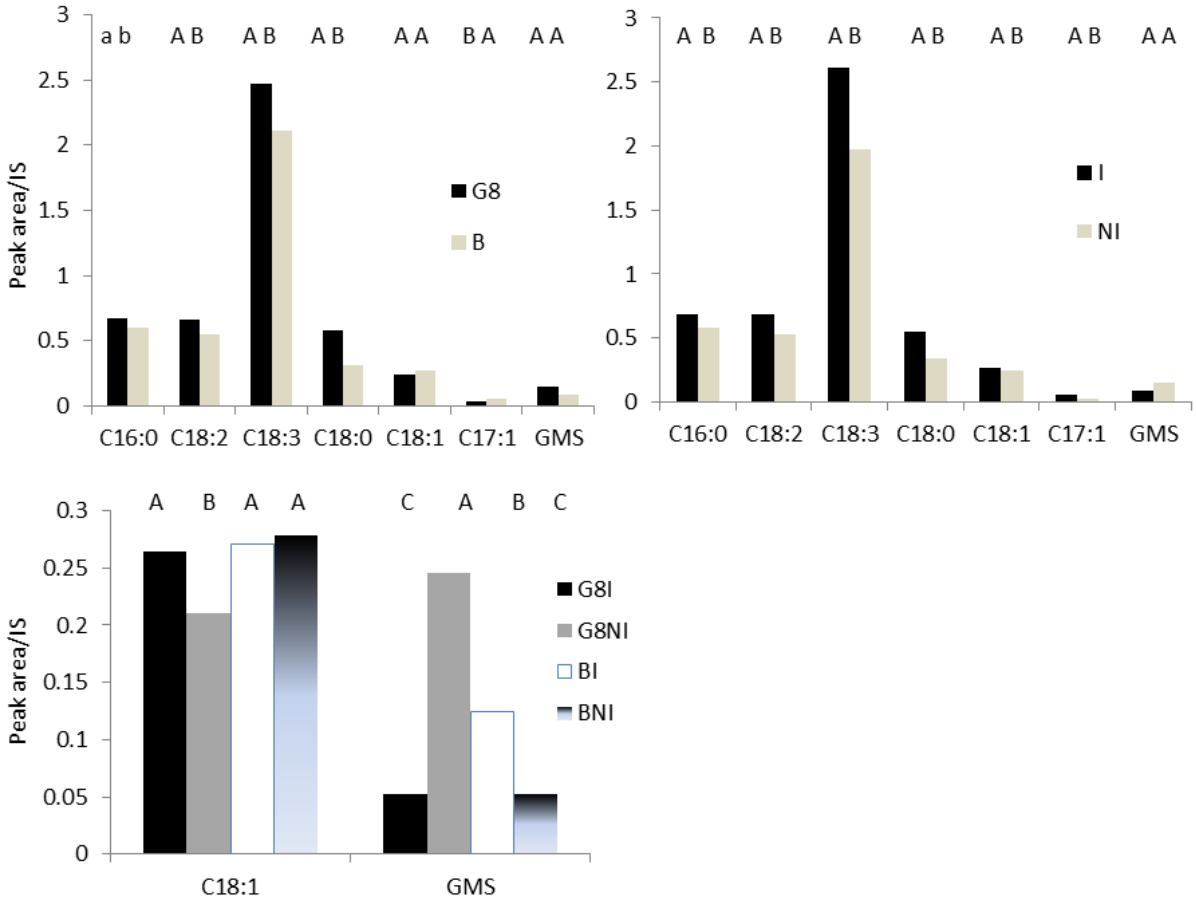
Acquisition Time (min)

Figure 2.


Peak area/IS
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2.5
2
1.5
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0.5
0
a b
A B
A B
A B
A A
B A
A A
C16:0
C18:2
C18:3
C18:0
C18:1
C17:1
GMS
G8
B
A B
A B
A B
A B
A B
A B
A A
I
NI
0.3
0.25
0.2
0.15
0.1
0.05
A
B
A
A
C
A
B
C
G8I
G8NI
BI
BNI
C18:1
GMS

Figure 3

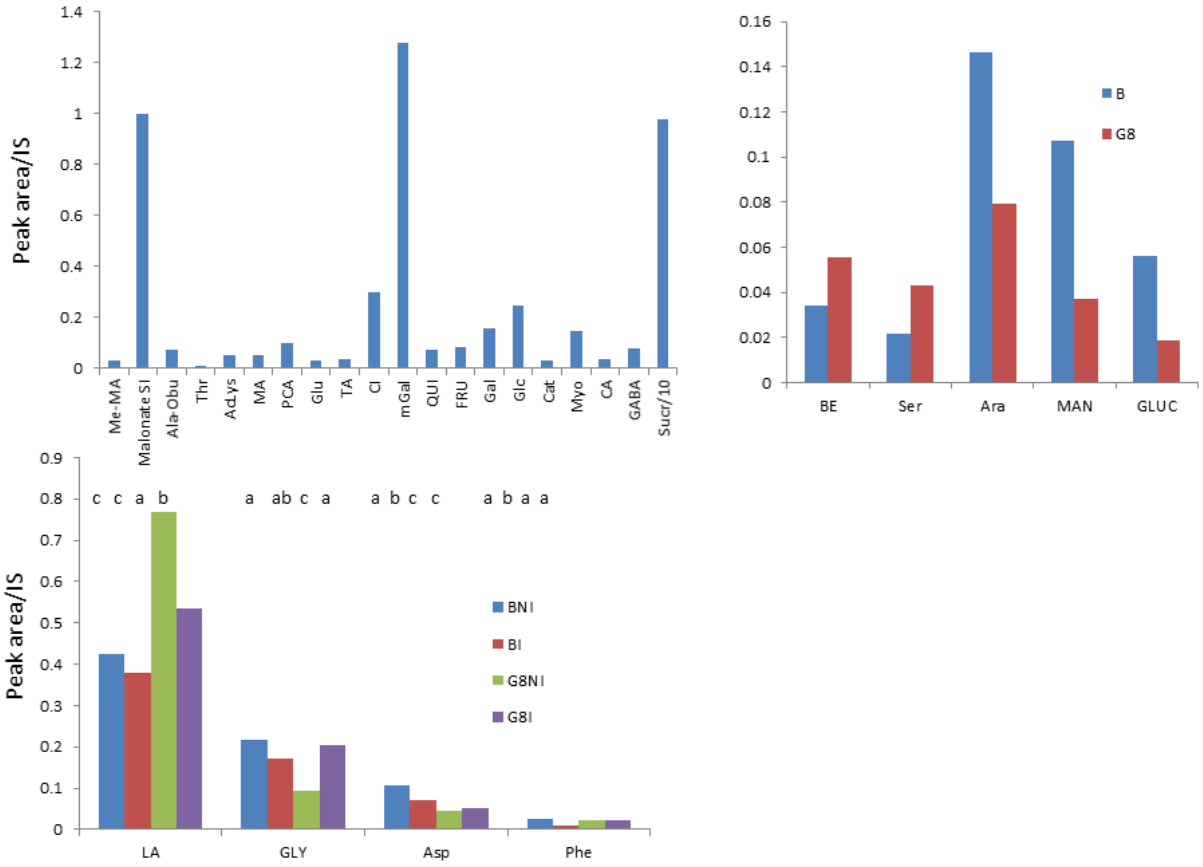
Peak area/IS
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Me-MA
Malonate SI
Ala-Obu
Thr
Ad.ys
MA
PCA
Glu
TA
CI
mGal
QUI
FRU
Gal
Glc
Cat
Myo
CA
GABA
Sucr/10
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
B
G8
BE
Ser
Ara
MAN
GLUC
Peak area/IS
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
c c a b
a ab c a
a b c c
a b a a
BNI
BI
G8NI
G8I
LA
GLY
Asp
Phe

Figure 4